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BIOLOGICAL AEROSOL TEST METHOD AND PERSONAL PROTECTIVE EQUIPMENT (PPE) DECON

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14. ABSTRACT

Capability to simulate surface deposition of contagious droplets from human respiratory secretions, developed for use in a BSL3 laboratory at the University of Nebraska Medical Center (UNMC), was used to quantitatively assess the effects of three decontamination methods [microwave/steam, moist heat, ultraviolet germicidal irradiation] on viable infectious influenza virus A/H5N1. This report describes this capability, including laboratory techniques and methods used to test and analyze samples.

UNMC will use this capability for aerosol-related research at to evaluate effectiveness of personal respirator protection devices. This effort evaluated technologies for disinfecting filtering facepiece respirators (FFRs) by aerosolizing, sampling, and analyzing viability of A/H5N1 virus on FFRs. Objectives included supporting and enhancing collection of quality data, characterizing and optimizing the aerosol test systems and supplementary data quantification using real-time polymerase chain reaction assays.

AFRL supplied two aerosol surface deposition test systems used to apply virus to the FFRs: These test systems were the Laboratory-Scale Aerosol Test System and Droplet Loading Device. Performance of both was validated in UNMC's BSL3 laboratory, first with colored dyes (to ensure equal distribution of aerosol across the system), then with viable virus, which verified system operations within design parameters.

AFRL's protocols were utilized throughout unless otherwise specified. FFRs were loaded with virus, subjected to one of the decontamination methods, then assayed for viable virus. All three treatments reduced viable virus concentrations by ≥ 4 $\log_{10}$ $TCID_{50}$.

15. SUBJECT TERMS

bioaerosol, decontamination, FFR, filter, H5N1, influenza, respiratory protection, reuse

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TABLE OF CONTENTS

LIST OF FIGURES

Page

LIST OF TABLES

1. EXECUTIVE SUMMARY

A capability designed to simulate surface deposition of contagious droplets from human respiratory secretions was developed for use in a BSL3 laboratory at the University of Nebraska Medical Center (UNMC). This capability was used to quantitatively assess the effects of three decontamination methods [microwave/steam (μ/S), moist heat (MH) and ultraviolet germicidal irradiation (UVGI)] on viable infectious influenza virus A/H5N1. This effort was in support of the Air Force Research Laboratory (AFRL) at Tyndall Air Force Base, Florida. Prior to this work, few/no laboratories possessed the expertise and capabilities to properly aerosolize and analyze the influenza A/H5N1 virus. This report describes the establishment of such capability, including the laboratory techniques and methods used in testing and analyzing samples.

The primary objective of this project was to establish the capability for conducting aerosol-related research at UNMC to evaluate the effectiveness of personal respiratory protective devices. A component of that effort was to conduct a study of technologies for disinfecting filtering facepiece respirators (FFRs) in support of a project at AFRL. This validation study consisted of using methods and equipment to aerosolize, sample, and analyze the viability of a low-pathogenic strain of influenza A/H5N1 virus on FFRs. Secondary study objectives were aimed at supporting and enhancing the collection of quality data, including characterizing and optimization of the aerosol test systems and supplementary data quantification through the use of real-time polymerase chain reaction (RT-PCR) assays.

For this study AFRL supplied two aerosol surface deposition test systems used to apply virus to FFR masks. These were the Laboratory-Scale Aerosol Test System (LSAT) and the Droplet Loading Device (DLD). Prior to testing, these two test systems were set up in UNMC's BSL3 laboratory for validation of their performance. First, colored dyes were used to ensure that the aerosol was equally distributed across all FFRs within the DLD. This was followed by experiments using viable virus. Results from these tests indicated that the system was operating within its designated parameters.

Protocols provided by AFRL were utilized for every aspect of the testing procedure from sample exposure to data analysis unless otherwise specified in the appendices. After exposure to virus, FFRs were subjected to one of three decontamination methods (μ/S, MH , UVGI radiation) and subsequently assayed for viable virus. Results indicate that all three decontamination methods reduced viable virus concentrations by ≥ 4 $\log_{10}$ $TCID_{50}$.

2. INTRODUCTION

2.1 Background

Many communicable diseases can be spread through droplets containing infectious agents. Such "contagious droplets" may expose susceptible individuals directly or contaminate environmental surfaces in the immediate vicinity and render them fomites, further enhancing the spread of disease. A variety of approaches have been developed to protect individuals from aerosolized infectious particles, including the FFR. Our national capability to deliver FFRs may not be able to meet demand during an outbreak of a severe infectious respiratory disease such as influenza. Therefore the question was raised as to whether FFRs can be decontaminated and reused. The issue of reuse raises numerous regulatory and safety issues; however, the basic scientific question is simply stated: can decontamination be achieved while preserving fit and function?

In a real-world environment particle size and composition of droplets influence the viability of aerosolized microorganisms exposed to environmental stresses. Inert components contained within the droplets or droplet nuclei may shield biological organisms from physical and chemical decontaminants. Further, each virus type may possess innate capabilities to survive based on physical composition of its outer membrane. Therefore, it is imperative to understand the factors that contribute to virus survivability on a variety of surfaces, including medically relevant materials. In response AFRL designed the Droplet Loading Device (DLD) test chamber to mimic real-world surface deposition of contagious droplets from human respiratory secretions.

2.2 Scope

The primary objective of this study was to evaluate decontamination methods (physical, chemical and self-decontaminating materials) applied to surfaces contaminated with virus-containing droplets. This work was primarily focused on influenza A/H5N1 virus but other respiratory viruses or surrogates could be used. To accomplish this, a test method was created to define the conditions for simulating respiratory droplets produced by humans during bouts of illness.

3.0 MATERIALS AND METHODS

3.1 Materials

3.1.1 Biological Organisms

Influenza A/H5N1 (VNH5N1-PR8CDC-RG) was acquired by UNMC from the Centers for Disease Control and Prevention (CDC) and transferred to a commercial laboratory for production in eggs. Virus was produced in eggs and recovered from allantoic fluid by Benchmark Biolabs (Lincoln, Nebr.). Virus titers were determined using a tissue culture median infectious dose assay ($TCID_{50}$) in Madin–Darby canine kidney cells (MDCK; ATCC CCL-34) by standard cell culture techniques (Appendix C). The stock concentration was titered by the vendor and by UNMC and found to be approximately 5.5 $\log_{10}$ $TCID_{50}$/mL. Electron microscopy performed at UNMC was used to image the A/H5N1 virus (Fig. 1) to confirm its morphologic identity.

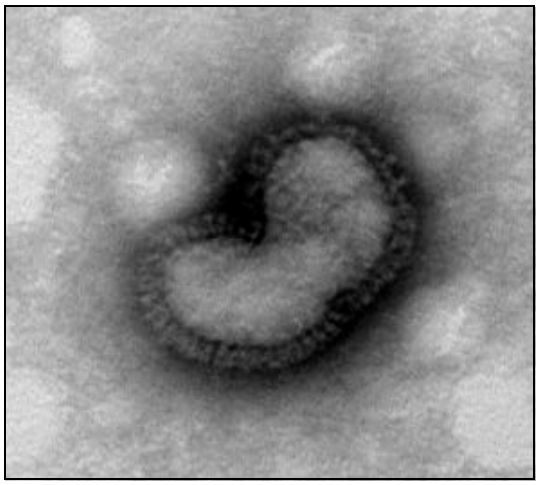

Figure 1. Transmission Electron Micrograph of Influenza A/H5N1

3.1.2 Filtering Facepiece Respirators

Two different commercially available models of FFRs were used in this study for direct filter comparisons between the two droplet deposition test systems, the 1860s and the 1870 (3M Corporation, St. Paul, Minn.). The FFRs used in this study consisted of electrostatically charged polypropylene filters (electret filter material) with low variability. Both the 3M 1860s and 1870 FFRs carry a National Institute for Occupational Safety and Health (NIOSH) N95 certification (42 CFR Part 84). The respirators were obtained through the commercial market.

The 1860s healthcare N95 FFR is designed to provide 95% efficient respiratory protection to a properly fitted wearer against oil-free aerosol particles 0.3 μm in diameter; removal efficiency is higher against particles that are significantly larger or smaller. This FFR is fluid resistant and

disposable (Fig. 2 a)). The 3M 1870 (Fig. 2 b)) is a flat-fold/three-panel, surgical, N95 respirator that is designed to resist splash and splatter of bodily fluids and infectious materials. These masks

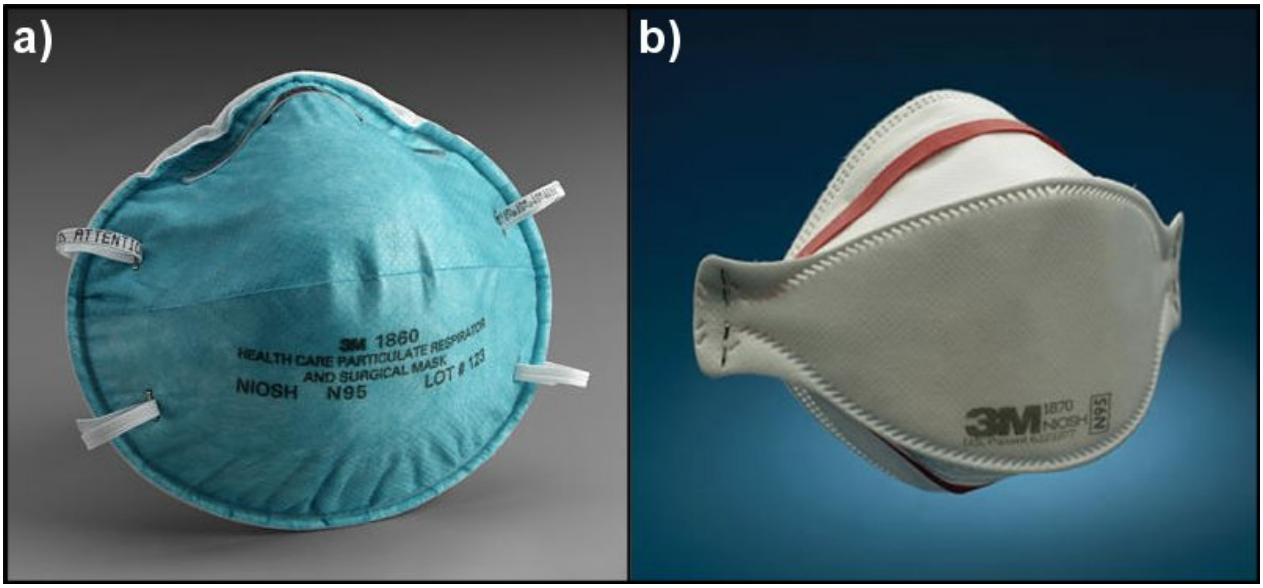

Figure 2. 3M Filtering Facepiece Respirators a) 1860s b)1870

are typically used by healthcare workers. Like the 1860s, the 1870 removes 95% of oil-free aerosol particles 0.3 μm in diameter, and a progressively larger fraction of particles significantly larger or smaller than the design dimension, from air that passes through the filter medium.

3.2 Methods

3.2.1 Test Method(s) Overview

The following method summary describes protocols and the apparatus for applying viral bioaerosols to FFRs, and subsequent laboratory operations used to quantitatively assesses efficiency of disinfection. The intent of these methods is to approximate respiratory secretions from humans by applying large droplets (~40 μm number median diameter) as well as smaller particle nuclei to surfaces. These methods have been designed specifically for the influenza virus, but other respiratory viruses and simulants could be delivered.

3.3 Test Methods: Droplet Loading Device (DLD)

3.3.1 Testing Procedures

The DLD was designed as a device capable of reproducibly loading predefined quantities of large droplet nuclei onto surfaces (see Fig. 3). This test system was used to simultaneously load an array of 12 FFRs (six 1860s and six 1870), each 5 cm from the edge and equally spaced relative

to the others, which were arranged on the rotating table of the droplet loader. The door to the DLD was sealed and the rotating table was adjusted to 3.0 revolutions per minute (rpm). Influenza A/H5N1 virus (25 mL of a dispersion containing ~5×10^5 $TCID_{50}$/mL) was loaded into the atomizer's reservoir, which contains a siphon tube. The siphon tube was connected to the air-atomizing nozzle (Paasche, Chicago, Illinois; Model SA 2000) and compressed air (~2 cfm) was delivered to draw the virus into the nozzle. Liquid flow to the nozzle was adjusted to deliver ~ 5 mL of viral suspension per minute. The FFRs were loaded with large droplets (~40 µm number median diameter (NMAD)) containing virus as the table revolved under the droplet stream delivered from above by the air-atomizing nozzle. After virus loading was complete, the compressed air was turned off and the remaining aerosol was allowed to settle in the chamber for 3 min before the test masks were removed. This procedure was repeated 18 times to complete the 216-article test matrix. After exposure each test filter was removed from the DLD. "Treated" filters were subjected to one of the three decontamination methods; untreated filters were used as controls.

Following the decontamination procedure, each filter was processed by cutting four 38-mm coupons from the mask and placing all four into a 50-mL conical tube containing 15 mL of Eagle's minimum essential medium (EMEM) (Hyclone Laboratories Inc, Logan, Utah). The coupons and medium were vortexed for 20 min. The EMEM was then decanted and used to make final dilutions, which were plated in quadruplicate to determine $TCID_{50}$ (Appendix C). Plates were stained and read 5–6 days after the initial plating for cytopathic effect (CPE). Additionally, samples were also assayed using RT-PCR.

3.3.2 System Description

The DLD test system (Figs. 3 and 4) was designed to mimic respiratory droplet transmission of viruses onto a surface. Operationally, the DLD is a settling chamber into which an air atomizing nozzle injects droplets that have a number median diameter of ~40 µm. Water evaporates from the droplets as they descend to the surface of the test samples, but the droplets retain sufficient water that they impact the test samples as liquid droplets. Uniformity of distribution of the droplets onto the test specimens is slightly improved by rotating the samples on a turntable at approximately 3 rpm. It is recognized that changes in surface shape may affect distribution onto the surface of the FFR.

3.3.3 System Design

The chamber of the DLD is a rectangular, stainless steel shell that has dimensions 24 in × 24 in × 30 in (l×w×h). The chamber has six ports on the top and bottom of the chamber to allow for introduction of aerosol and the exit of air laden with undeposited particles, respectively. The ports are 3/8-in NPT threaded openings spaced 6 in from the center of the chamber. The ports are spaced 6 in apart in a circular pattern. The rear panel of the chamber also contains two 3/8-in NPT threaded ports, which are used to install humidity and temperature probes. The chamber contains an access door (22 in × 13 in) located 5 in from the bottom of the chamber. A fractional-horsepower DC gear motor mounted in the bottom of the chamber is attached to a 22-in-diameter circular turntable that is perforated with 1/8-in holes. The turntable is positioned 6 in above the bottom of the chamber. The motor is wired to a DC speed controller that is used to set the speed of the turntable. An air atomizing nozzle (Passche, Chicago, Illinois; part number SA 2000), is

mounted into a 3/8-in diameter fitting using epoxy. The nozzle is fitted into one of the top chamber ports closest to the access door. The other ports on the top of the chamber are sealed with set screws. One port on the bottom of the chamber is fitted with high-efficiency particulate air (HEPA) filters.

Figure 3. Droplet Loading Device

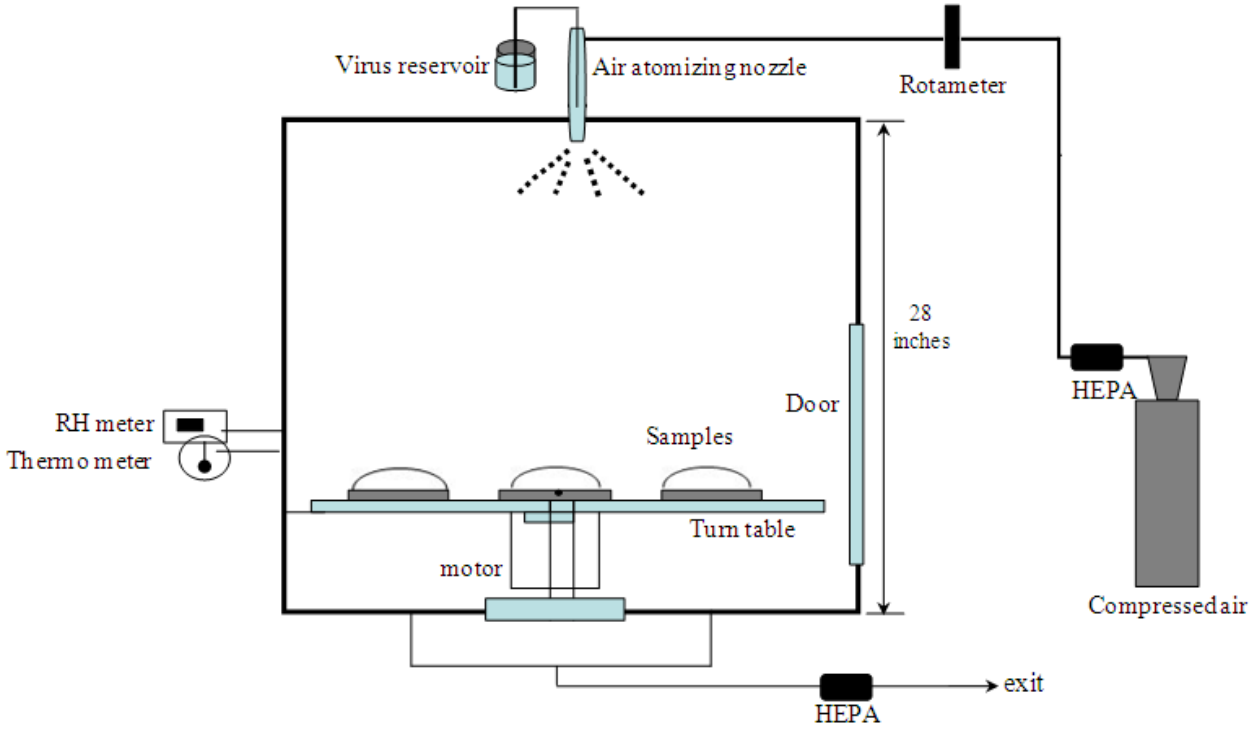

Figure 4. Schematic of the Droplet Loading Device

3.3.4 Validation of Test System

3.3.4.1 Requirements

A virus loading validation was conducted using two models of FFRs; the 3M 1870 and the 3M 1860s. These tests were used to ensure complete and uniform coverage of aerosolized virus over the entire surface of the test respirators.

3.3.4.2 Droplet Characteristics

The droplets generated for this method were measured by AFRL to have a NMAD of ~40 µm. This was established before delivery and UNMC performed no further droplet validation tests.

3.3.4.3 Sample Loading Uniformity

In initial experiments a dye was used to characterize the aerosol coverage resulting from the aerosol generator. The aerosol generator was loaded with 25 mL of an aqueous solution containing a colored dye that was atomized according to the standard procedure while the DLD's turntable was rotating. White copy paper was used to cover the entire top of the turntable, which allowed for the visualization of the aerosol generator's coverage pattern. After a satisfactory coverage pattern was observed infectious influenza A/H5N1 virus was delivered in subsequent experiments performed to measure sample-to-sample variation.

3.3.4.4 Sample-to-Sample Variation

The variability of virus loading for multiple samples loaded for a single test was validated using FFRs exposed to A/H5N1 virus. A total of six FFRs of each model were exposed to A/H5N1 using our standard testing protocols. Results from validation tests confirmed that the loading was within the quality parameters (± 0.5 logs using the $TCID_{50}$ assay) described in the DLD testing protocol (Appendix C). The presence of the virus on the FFRs was also detected using RT-PCR. These data verified that the FFRs were being uniformly loaded with aerosolized virus particles.

3.4 Test Methods: Laboratory-Scale Aerosol Test System (LSAT)

3.4.1 Testing Procedures

A six-jet Collison nebulizer (BGI Inc., Waltham, Massachusetts) was loaded with A/H5N1 virus at a concentration of 6.0 $\log_{10}$ $TCID_{50}$/mL and attached to the test system. The LSAT media holder was loaded with sample filters one at a time, face up with the metal nosepiece oriented away from the axis of rotation. Using a HEPA-filtered air supply aerosol generation was initiated and maintained for 10 min at 30 psi air pressure. After exposure each test filter was removed from the LSAT. Treated filters were exposed to one of the three decontamination methods while the non-treated filters were used as controls.

Following the general procedure described in section 3.3.1, four 38-mm coupons were cut from each mask, placed as a group into a 50-mL conical tube containing 15 mL of EMEM and vortexed for 20 min. The EMEM was decanted, and serial dilutions were plated in quadruplicate to determine $TCID_{50}$ as described in Appendix C. Plates were stained and read 5–6 days after plating for cytopathic effect (CPE), and samples were assayed using RT-PCR.

3.4.2 System Description

The LSAT, which is schematically illustrated in Figure 5, was originally designed and built to measure the particle removal efficiency of several types of filter media. The LSAT is based on the design of a small-scale vertical test system. However, the unit employed to perform this study was modified so that the LSAT has a horizontal chamber rather than a vertical chamber. This modification was necessary to permit placement of the test system inside a biological safety cabinet to provide the advanced containment necessary for experiments with aerosolized respiratory pathogens.

3.4.3 System Design

The LSAT was constructed from modular 10.2-cm (4-in) nominal diameter stainless steel tubes and fittings. The system was stacked in a configuration that located the aerosol generation and dilution system directly above the main body of the system (Figs. 5 and 6). Challenge aerosols were generated using a Collison nebulizer supplied with HEPA-filtered air at 30 psi. After the aerosol was generated, it traveled through a porous tube diluter (Mott Corp., Farmington, Connecticut) in which HEPA-filtered air is added into the system to ensure the formation of solid, “dry” particles (*droplet nuclei*). The droplet nuclei then pass through a krypton-85 charge

neutralizer (TSI model 3012; TSI Inc., Minneapolis Minnesota), which decreased the charge distribution induced on the particles during nebulization to a Boltzmann equilibrium before they reached the test filter. After passing through the test filter, the airflow exits the system through a HEPA filter and a calibrated mass flow meter.

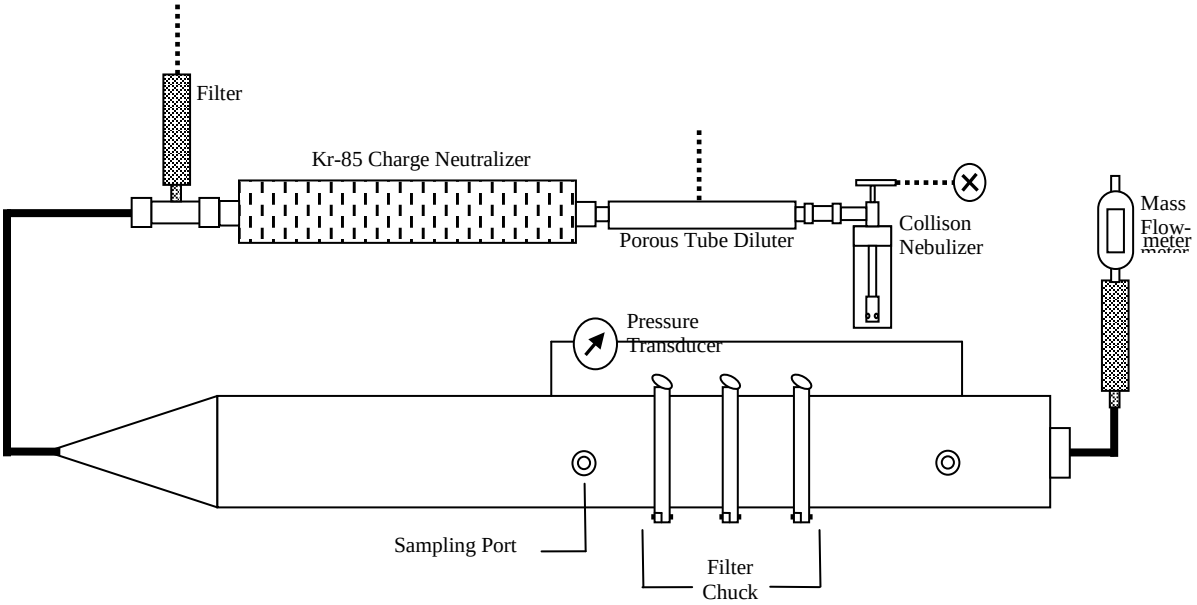

Figure 5. Schematic of the Laboratory-Scale Aerosol Test System

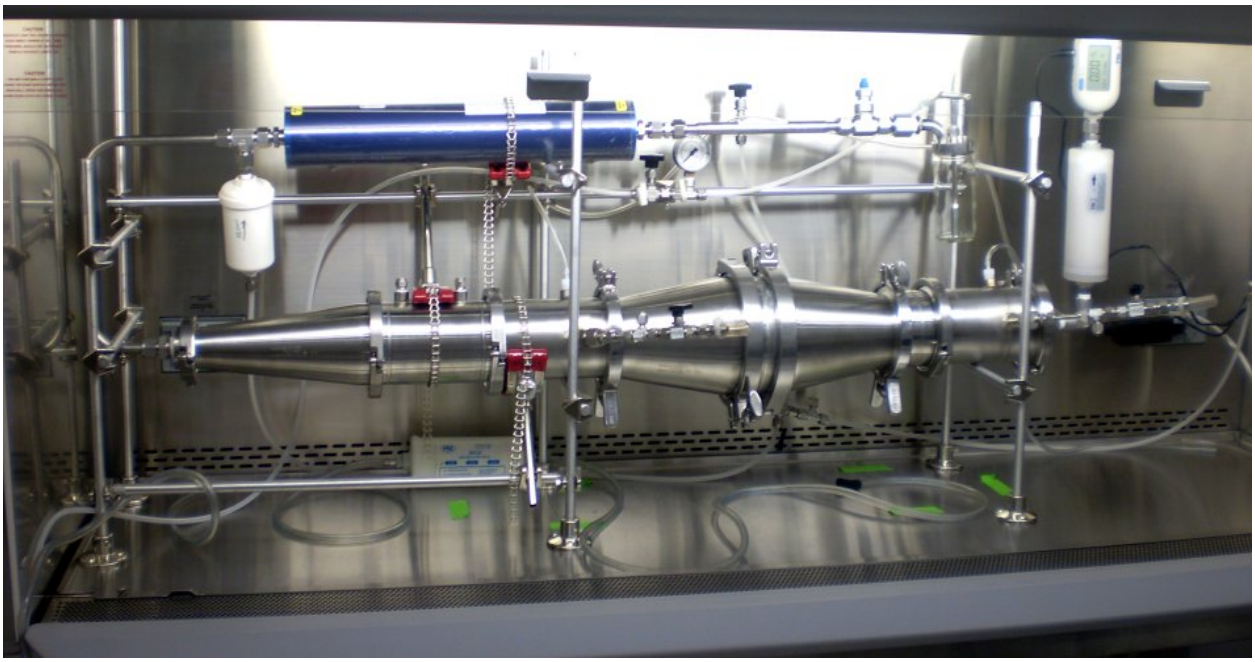

Figure 6. Laboratory-Scale Aerosol Test System

During initial testing it was determined that an insufficient amount of virus was being recovered. Reconfiguration of the test system to eliminate two 90-degree turns provided for a straight-line connection between the nebulizer and respirator, which reduced virus particle losses and allowed for a more-accurate determination of $TCID_{50}$.

3.4.4 Validation

Orifice calibration plate airflow standards were fabricated for each test system, individual chokes being sized to fit into each of the filter medium chucks. Aluminum sheet metal circles with a thickness of 0.78 mm were perforated with five 0.56-cm diameter holes in a square die pattern with a 5-cm diagonal. The pressure drop across each of these plates was correlated with volume flow as measured with an American Meter Company model DTM-325 Dry Test Meter (Mac Systems, Inc., Tullytown, Pennsylvania). This method was used to periodically check the volume air flow calibration of the TSI Model 4043E mass flow meter.

3.5 Filter Decontamination Procedures

The disinfection protocols subjected the FFRs to one of three disinfection techniques (μ/S, MH, UVGI) before plating. Control samples were incubated under identical conditions but were not exposed to the disinfection protocols prior to plating. Virus particles were eluted from the disinfected and control FFRs, and viability was assessed by $TCID_{50}$ end-point assay (Appendix C). The virucidal activity of the disinfection procedure is determined from the log difference in viability between the treated (decontaminated) and untreated (control) FFRs.

For each filter trial, three of the A/H5N1-contaminated FFRs were decontaminated and the other three served as positive controls. To minimize loss of A/H5N1 viability due to normal decay, decontamination studies were performed immediately after loading each FFR. Control FFRs were incubated at room temperature for the same times as FFRs treated by the decontamination technologies.

3.5.1 Moist Heat (MH)

A 6-L sealable container (17 cm (h), 19 cm (w), 19 cm (l)), was filled with 1.0 L of tap water. A plastic support rack was placed in the water to isolate the FFR from the liquid. Prior to the test, the container was warmed in a 65 ºC ± 5 ºC oven for a minimum of 3 h. The container was removed from the oven and an A/H5N1-contaminated FFR was placed on the rack. The containers were sealed and returned to the oven for 20 min.

3.5.2 Microwave/Steam (μ/S)

Two 7.6-cm (h) x 12-cm (w) x 8-cm (l) plastic reservoirs with perforated tops (96 holes [6 mm diameter] equally spaced over the entire surface) were filled with 50 mL each of tap water at room temperature. The reservoirs were placed together and the A/H5N1-contaminated FFR was set atop the center of the assembly, with the exterior of the FFR resting on the surface of the reservoir. The reservoir assembly and FFR were loaded into the center of a Panasonic Inverta

1250-W microwave oven and irradiated at full power for 2 min. Following treatment, the reservoir was replenished with fresh water and the next FFR was processed.

3.5.3 Ultraviolet Germicidal Irradiation (UVGI)

A 120-cm 80-W UVGI (254-nm) lamp (Ultraviolet Products, Upland, Calif.) was adjusted to a height of 25 cm. Output from the lamp was measured using a radiometer (Ultraviolet Products, Upland, Calif.); over the range in which the FFR was exposed to UV irradiation, the dose varied from 1.6 mW/cm^2 to 2.2 mW/cm^2. The exterior surface of H1N1-contaminated FFRs was irradiated for 15 min, which provided an average dose of 18 kJ/m^2. The local exposure varied over the FFR due to the curved shape of the device.

3.6 Biological Sampling

3.6.1 Virus Extraction

Four 38-mm diameter circular coupons were cut from four quadrants of each FFR using a standard punch. The four coupons were placed in a 50-mL conical tube containing 15 mL of serum-free EMEM supplemented with 1% L-glutamine (Sigma–Aldrich, St. Louis, Missouri) and 1 % pen/strep (sf-EMEM-p/s-g). The samples were mixed for 20 min at maximum speed using a multi-tube vortex mixer (VWR Scientific, West Chester, Pennsylvania).

3.7 Virus Detection Methods

3.7.1 Determination of Viable Virus Titer by $TCID_{50}$

Viable A/H5N1 virus contained in the extracts was quantified by $TCID_{50}$ end-point assay using the MDCK cell line obtained from Diagnostic Hybrids (Athens, Ohio). Maximization of sensitivity being desirable, the assay was performed using the entire extract for the decontaminated samples. The extract for the control FFRs was serially diluted (1/10) in the sf-EMEM-p/s-g and all dilutions were delivered in quadruplicate into the 24-well plates. The plates were incubated for 5–6 days a 5% CO_2 atmosphere at 37 ºC prior to visualizing CPE.

Confluent 24-well plates containing MDCK cells were obtained from Diagnostic Hybrids, Inc. (Athens, Ohio), and maintained following protocol C-1 (in Appendix C). Additionally, when necessary, cell culture was performed in house using procedure C-2, outlined in Appendix C. Briefly, MDCK cells were propagated in EMEM supplemented with L-glutamine, penicillin and streptomycin and heat-inactivated fetal bovine serum. Cells were split as needed from large T-75 flasks into 24-well plates. Wells were seeded with approximately 30,000–60,000 cells and allowed to double until reaching 85–95% confluency, at which time they were used for viral $TCID_{50}$ assay(s). The Spearman–Karber formula (Appendix C, C-3) was used to determine the concentration of viable virus per mL of extract, expressed in units of $\log_{10}$ $TCID_{50}$/mL.

3.7.2 Determination of Absolute Virus Titer by RT-PCR

Real-time polymerase chain reaction (RT-PCR) was used as a secondary analysis to provide additional information about the effectiveness of the decontamination treatments. This non-culture assay is the most-sensitive technique for detection and quantification of genetic material

currently available. However, the detection of genetic material does not necessarily indicate the presence of viable organisms. Therefore it may be used to document the presence of virus when culture results are negative.

RNA was extracted using a QIAamp viral RNA extraction kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. RNA was recovered in 15 µL (final volume) of elution buffer (supplied by the manufacturer) and either used immediately or stored at -80 °C. Quantification of the extracted RNA was accomplished spectrophotometrically using a NanoDrop ND-1000 (Saveen Werner, Limhamn, Sweden) spectrophotometer. RNA amplification of the hemagglutinin (HA) viral protein target was carried out according to the CDC protocol (Appendix D) using Invitrogen's SuperScript III Platinum One-Step qRT-PCR System (cat no. 11732-088), which combines the reverse transcription and amplification steps.

Assay conditions for quantification of extracted RNA were optimized in a Roche LightCycler 480 Real-Time PCR System (Roche Diagnostics). The Superscript III Platinum One-Step Mastermix reaction components were prepared to the indicated end concentration: 5.5 µL nuclease-free water, 0.5 µL H5a-F forward primer (SO3307;CDC), 0.5 µL H5a-R reverse primer (SO3308;CDC), 0.5 µL H5a-P probe (SO3294;CDC), SuperScript III RT/Platinum *Taq* mix (55176; Invitrogen) and 12.5 µL 2x PCR master mix. For each sample, 20 µL of the complete LightCycler mastermix and 5 µL of extracted RNA (25 µL total) were loaded into each well of a 96-well plate. The plate was then loaded into the LightCycler. Samples were run in triplicate for each dilution and presented as the mean cycle threshold (Ct) value.

The RT-PCR thermocycling parameters were as follows: initial *c*DNA synthesis 50 °C for 30 min; then denaturation at 95 °C for 2 min, followed by 45 cycles of 15 s of denaturing at 95 °C, 30 s of annealing at 55 °C, and 30 s of extension at 72 °C and finally a cooling step to 40 °C. Total run time was approximately 2 h.

4. RESULTS AND DISCUSSION

4.1 DLD Test System: Viable Virus Concentration Data

The average concentration of influenza A/H5N1 virus recovered from the untreated FFRs for each test ranged from 4.65–5.17 $\log_{10}$ $TCID_{50}$/coupon over all 54 masks (Tables 1 and 2). Each $\log_{10}$ $TCID_{50}$ cell within the tables (Tables 1 and 2) is the average of three replicate samples. Each mask was tested three times for a total of nine replicates per respirator. Decontamination procedures resulting in viable counts (CPE) below the end-point assay's detection limit (1 $\log_{10}$ $TCID_{50}$) were calculated as zero. Therefore the reduction of virus titer between the untreated samples and samples exposed to a decontamination procedure was reported as an "absolute log reduction." Gross physical observations of FFRs following decontamination treatment showed no obvious signs of deterioration or deformation.

Table 1: Decontamination Results for 3M 1870 and 1860s Respirators Exposed to A/H5N1 Aerosol Using the Droplet Loading Device

	UVGI (~1.8×10^4 J/m^2)		Microwave/Steam		Moist heat (62 °C/85% RH)	
	Neg. control	Treated	Neg. control	Treated	Neg. control	Treated
3M 1870	4.93	*BDL	4.76	*BDL	4.68	*BDL
Log_{10} $TCID_{50}$ (per coupon)	4.68	*BDL	4.76	*BDL	4.68	*BDL
	4.34	*BDL	4.84	*BDL	4.59	*BDL
Average	4.65	***NA	4.79	***NA	4.65	***NA
Absolute Log Reduction**	**4.65**		**4.79**		**4.65**	
Lower 95% CI	5.39		4.90		4.78	
Upper 95% CI	3.91		4.67		4.52	
	Neg. control	Treated	Neg. control	Treated	Neg. control	Treated
3M 1860s	4.51	*BDL	4.76	*BDL	4.68	*BDL
Log_{10} $TCID_{50}$ (per coupon)	4.68	*BDL	4.84	*BDL	4.68	*BDL
	4.43	*BDL	4.84	*BDL	4.51	*BDL
Average	4.54	***NA	4.81	***NA	4.62	***NA
Absolute Log Reduction**	**4.54**		**4.81**		**4.62**	
Lower 95% CI	4.86		4.93		4.86	
Upper 95% CI	4.22		4.70		4.38	

* Below Detection Limit; for log reduction a detection limit of 1 $\log_{10}$ $TCID_{50}$ was used. Therefore values for BDL were calculated as zero.
** Absolute Log Reduction = the average of the neg control group – the treated group.
***NA = not applicable

Table 2. Decontamination Results for 3M 1870 and 1860s Respirators Exposed to H5N1 Aerosol Using the Laboratory-Scale Aerosol Test System

	UVGI (~1.8×10^4 J/m^2)		Microwave/Steam		Moist heat (62 °C/85% RH)	
	Neg. control	Treated	Neg. control	Treated	Neg. control	Treated
3M 1870 Log_{10} $TCID_{50}$ (per coupon)	4.93	*BDL	5.01	*BDL	4.93	*BDL
	4.84	*BDL	5.26	*BDL	4.84	*BDL
	5.26	*BDL	5.18	*BDL	5.18	*BDL
Average	5.01	***NA	5.15	***NA	4.98	***NA
Absolute Log Reduction**	**5.01**		**5.15**		**4.98**	
Lower 95% CI	5.56		5.46		5.41	
Upper 95% CI	4.46		4.83		4.55	
	Neg. control	Treated	Neg. control	Treated	Neg. control	Treated
3M 1860s (Log_{10} $TCID_{50}$ per coupon)	5.01	*BDL	5.43	*BDL	5.68	*BDL
	5.18	*BDL	5.18	*BDL	5.34	*BDL
	4.93	*BDL	4.76	*BDL	5.01	*BDL
Average	5.04	***NA	5.12	***NA	5.34	***NA
Absolute Log Reduction**	**5.04**		**5.12**		**5.34**	
Lower 95% CI	5.35		5.96		6.18	
Upper 95% CI	4.72		4.28		4.51	

* Below Detection Limit; for log reduction a detection limit of 1 log_{10} $TCID_{50}$ was used. Therefore values for BDL were calculated as zero.
** Absolute Log Reduction = the average of the neg control group – the treated group.
***NA = not applicable

The DLD untreated (control) samples for the 1870 respirators (27 masks total) averaged a CPE titer of 4.69 log_{10} $TCID_{50}$/coupon (Table 1) and the 1860s respirator controls (27 masks total) averaged 4.65 log_{10} $TCID_{50}$/coupon (Table 2). The variability in titer between replicates is within the enumeration error of ± 0.5 log_{10}. Results indicate little variation in the day-to-day application of virus. The log_{10} $TCID_{50}$ reduction for the 1870 respirators exposed to decontamination methods in the DLD were as follows; UVGI 4.65, μ/S 4.65, MH 4.79. The log_{10} $TCID_{50}$ reduction for the 1860s respirator was UVGI 4.62, μ/S 4.54, MH 4.81. All three decontamination methods resulted in an absolute log reduction of ≥ 4.5 logs for both respirator models.

4.2 DLD Test System: Total Virus Concentration Data

The RT-PCR “total virus concentration” data in Table 3 indicate that viral genome was still present and intact enough to be amplified. Each dilution represents a mean value of nine replicates. All values are reported as cycle threshold (Ct) values, i.e., the number of amplification cycles needed to cross a background threshold.

Table 3: RT-PCR Ct values of FFRs exposed to H5N1 aerosol using the DLD

Untreated (Control) Group								
Microwave/Steam 3M 1870 (3 replicates)			Moist Heat 3M 1870 (3 replicates)			UVGI Radiation 3M 1870 (3 replicates)		
Dilution	Average	CoV (%)	Dilution	Average	CoV (%)	Dilution	Average	CoV (%)
0	16.14	6.63	0	16.88	2.71	0	16.39	4.42
1	19.83	4.94	-1	20.23	4.01	-1	19.36	2.69
2	23.28	3.76	-2	23.75	3.21	-2	22.71	1.88
3	26.51	3.68	-3	27.04	2.49	-3	26.17	1.76
4	29.77	2.94	-4	30.43	2.18	-4	29.11	1.07
5	32.89	3.48	-5	33.69	2.20	-5	32.74	0.46
3M 1860s (3 replicates)			3M 1860s (3 replicates)			3M 1860s (3 replicates)		
Dilution	Average	CoV (%)	Dilution	Average	CoV (%)	Dilution	Average	CoV (%)
0	16.98	5.66	0	17.36	3.95	0	16.59	1.67
1	20.61	4.53	-1	20.86	3.84	-1	19.75	1.65
2	25.20	6.27	-2	24.54	3.05	-2	23.21	3.68
3	27.16	3.86	-3	27.52	3.36	-3	26.39	3.47
4	30.54	3.28	-4	30.97	2.73	-4	29.52	2.78
5	33.81	3.61	-5	34.38	3.26	-5	32.89	2.29
Treated (Decontamination) Group								
Microwave/Steam 3M 1870 (3 replicates)			Moist Heat 3M 1870 (3 replicates)			UVGI Radiation 3M 1870 (3 replicates)		
Dilution	Average	CoV (%)	Dilution	Average	CoV (%)	Dilution	Average	CoV (%)
0	21.80	5.87	0	22.98	5.62	0	30.67	2.97
1	24.91	5.64	-1	26.07	5.38	-1	33.65	2.60
2	28.06	4.41	-2	29.46	4.22	-2	36.60	2.39
3	31.25	4.65	-3	32.86	3.46	-3	*37.23	1.67
4	34.68	4.28	-4	36.24	2.93	-4	*37.42	
5	*37.21	1.76	-5	*38.05	3.82	-5	*ND	
3M 1860s (3 replicates)			3M 1860s (3 replicates)			3M 1860s (3 replicates)		
Dilution	Average	CoV (%)	Dilution	Average	CoV (%)	Dilution	Average	CoV (%)
0	19.81	4.01%	0	20.05	2.45	0	29.10	5.60
1	23.20	4.34%	-1	23.29	1.06	-1	32.29	4.71
2	26.51	3.82%	-2	26.64	0.43	-2	35.32	5.25
3	29.85	3.44%	-3	30.32	1.92	-3	37.09	0.37
4	33.03	3.28%	-4	33.60	1.69	-4	*ND	
5	*37.06	3.57%	-5	*37.52	0.89	-5	*ND	

*ND = Not detected. .

There was a clear shift in the number of amplification cycles needed to cross the cycle threshold after each decontamination procedure was performed. The μ/S and MH appeared to perform similarly, averaging almost five additional amplification cycles beyond the untreated control to first detect the eluted A/H5N1 virus. The UVGI treatment took approximately 14 more amplification cycles to first detect the virus. This was nine cycles amplification more than the other two decontamination methods and most likely a result of UVGI's ability to denature genetic material. The maximum cutoff for virus detection with a high degree of confidence was defined as 37 amplification cycles. Any amplification occurring beyond 37 cycles was considered unreliable and at the point of the assay's detection limit. Therefore RT-PCR samples requiring ≥37 cycles are considered to have no virus present and reported as not detected (ND).

Ct values can be converted into log dilutions of virus. Each 3.3 Ct cycles is equal to one log dilution of virus. The log difference is defined as [Ct (untreated samples) - Ct (treated samples)]/3.3, 3.3 being the number of Ct cycles equivalent to one log in sample dilution. Therefore the log reduction in virus titer is approximately 1.5 logs for μ/S and for MH, and 2.7 logs for UVGI.

A definite trend is observed in the Ct data between mask types. This could suggest greater retention of virus by the 1870 respirator. However, measurements of culturable counts (Tables 1 and 2) suggest that although the virus is detected by molecular methods, viability is below culturable detection limits, defined in this study as 1 $\log_{10}$ $TCID_{50}$/mL.

4.3 LSAT Test System: Viable Virus Concentration Data

The LSAT untreated (control) samples for the 1870 respirators (27 masks total) averaged a CPE titer of 5.05 $\log_{10}$ $TCID_{50}$/coupon and the 1860s respirator controls (27 masks total) averaged 5.17 $\log_{10}$ $TCID_{50}$/coupon (Table 4). The variability in titer between replicates is within the enumeration error of ± 0.5 $\log_{10}$. Results indicate little day-to-day variation in the application of virus. The $\log_{10}$ $TCID_{50}$ reduction for the 1870 respirators exposed to decontamination methods in the LSAT were as follows: UVGI 5.01, μ/S 5.15, MH 4.98. The $\log_{10}$ $TCID_{50}$ reduction for the 1860s respirator was UVGI 5.04, μ/S 5.12, MH 5.34. All three decontamination methods resulted in an absolute log reduction of ≥ 4.5 logs for both respirator models.

5. CONCLUSIONS

Both the LSAT and the DLD were successfully installed at UNMC and both achieved reproducible contamination of N95 respirator surfaces with low-pathology A/H5N1 virus at concentrations large enough to validate the efficacy of three practical disinfection methods—μ/S, MH and UVGI—for disinfection and potential reuse of two disparate models of disposable N95 half-face particle-filtering respirators. In a series of tests that challenged two commercial N95 half-mask FFRs with A/H5N1 and then applied these three technologies, earlier results from challenges of the same FFR models with A/H1N1 and application of the same three disinfection methods were shown to be the same within the accuracy of the experiments.

For all three methods tested disinfection was complete within the detection limits of both the $TCID_{50}$ end-point method and CPE determinations—both direct measures of viability. In contrast, RT-PCR analysis—which measures the number of amplifiable nucleic acid fragments—showed that the relatively mild μ/S and MH treatments increased the Ct threshold by only ~5 cycles, whereas UVGI increased the detection threshold by ~14 cycles—almost three orders of magnitude. This appears to indicate that the free-radical mechanism activated by UVGI acts at least in part directly on the nucleic acids, causing crosslinks and other decomposition processes that drastically decrease the population of amplifiable fragments. For microbes more resistant than influenza virus to disinfection UVGI is likely to be a more-effective sterilization treatment; however, for the viruses here tested the antimicrobial efficiency of the three methods is seen to be the same.

Whereas the question of reusability of disinfected FFRs remains open, the aims of the project have been realized—the LSAT and DLD taken with techniques acquired in the course of this effort will support the desired development of a long-term program of viable aerosol challenge testing of either inert or reactive individual respiratory protective equipment

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APPENDIX A

PROTOCOL FOR OPERATION OF DROPLET-LOADING DEVICE

A-1. Test Procedure

1. Add 25 mL of influenza A/H5N1 virus suspension (~5.5 $\log_{10}$ $TCID_{50}$ /mL) to the aerosol nebulizer's reservoir and attach it to the nebulizer.
2. Place test articles into the settling chamber so they are equally spaced relative to one another. Space control and test samples alternately and close the door.
3. Set the turntable to ~3 rpm.
4. Turn on the compressed air and increase the flowrate until the nebulizer is primed.
5. Once the nebulizer is primed, set the air flowrate to ~2 cfm as indicated by the rotameter.
6. Expose the samples until the entire volume (25 mL) in the nebulizer's reservoir is consumed.
7. Turn off the pressure to the air atomizing nozzle.
8. Open door, lower sash on secondary containment hood and allow remaining aerosol droplets to settle for 3 min.
9. Remove the samples from the droplet loader and perform decontamination tests.

APPENDIX B

PROTOCOL FOR OPERATION OF THE LABORATORY-SCALE AEROSOL TEST SYSTEM

B-1. Test Procedure

1. Before starting the system make sure the overflow valve is open on the LSAT (to avoid over-pressurizing the system).
2. Turn on the air generation system main power, and press "reset" to turn off the alarm.
3. Turn on the compressor switch; set air pressure to 45 psi on the supplemental air (R3) gauge.
4. Turn on the main valve attached to the air generator supply line, (supply 1).
5. The secondary valve on the supply line inside the bio-safety hood (supply 2) controls airflow into the porous tube diluter of the LSAT
6. Open the valves on the LSAT, open the system valve, close the overflow valve
7. Set the airflow with the supply 2 valve. Adjust the valve while watching the flow meter to select the desired flow rate.
8. Open the over-flow value, close the system valve and check the flow meter, which should read zero.
9. If it does not, set the flow meter to read zero.

Loading the Collison nebulizer

1. Check the *O*-ring gasket and top seal gasket for structural integrity, and inspect the glass jar for cracks and for pits at top seal.
2. Hand tighten the Collison jet onto the nebulizer tube.
3. Load a volume of 10 mL or more of the biological challenge at the desired dilution into the glass reservoir, seal the jar snugly, wrap Parafilm® around the top connection, attach the air line, and attach the nebulizer to the LSAT. Snug the connection with a wrench.
4. Loading H5N1 at 30 psi produces a flow of ~19 to 20 Lpm without introduction of additional air into the system.

Loading respirator/filter into chamber

1. Glue respirators onto individual metal ring holders.
2. Select and install gaskets of the proper size to seal connections.
3. Using metal clamps, seal the respirator into place. Orient the respirator the same way each time, and tighten clamps with a screwdriver to ensure gaskets have sealed properly.

Flow rate settings

1. Set the Collison nebulizer airflow at the desired rate.
2. Leave the flow at this rate for 10 min to equilibrate the system.
3. Following 10 min of equilibration, open the system valve and close the overflow valve.
4. Check the flow meter. Record flow rate, relative humidity, temperature and pressure drop.

Termination of loading cycle and removal of filter for evaluation

1. At the end of the loading time open the overflow valve and shut off the system valve.
2. If additional runs are planned allow the Collison to continue to run into the overflow valve.
3. Loosen metal clamps holding the respirator in place and slide them to one side of the respirator holder.
4. Remove the metal ring containing the respirator, carefully supporting it.
5. Remove the respirator from the glue seal by pushing on the seal at it weakest point.
6. Remove the respirator from the ring and transfer it into a container for transport outside the hood.
7. Seal the respirator in the container to be transported to the decontamination method.
8. Load a new respirator as above, and replace and tighten the metal clamps to seal it into the LSAT chamber.
9. Repeat the aerosol loading procedure.

Shutdown

1. Remove the Collison nebulizer from the LSAT. Save any nebulization fluid for analysis of virus survival after aerosolization.
2. Remove the Collison nebulizer and attach the tubing cap to the opening into the LSAT.
3. Open the main system valve; close the by-pass valve.
4. Turn on air flow at approximately 20 to 25 Lpm and allow dry air to flow through the system for 3 h to decontamination the LSAT.
5. When flushing is completed turn off the dry air supply to the porous tube diluter. This stops all air into the LSAT.
6. Power off the vacuum pump and the compressed air supply.

APPENDIX C

$TCID_{50}$ ASSAY PROTOCOL

E.1 Plating Method #1

Confluent 24-well plates of monolayer Madin–Darby Canine Kidney (MDCK) cells

Confluent 24-well plates were ordered from Diagnostic Hybrids (Athens, OH). Once received, plates were placed in a CO_2 incubator 24 h before use. Plates are also produced in house with MDCK cells ordered from Diagnostic Hybrids (see Plating Method #2).

Titrate virus stocks

Make tenfold serial dilutions of the virus-containing samples in sf-EMEM.

Wash wells three times with 1xPBS

Add 1.0 mL of 1xPBS to each well. Rinse and discard. Repeat this wash 2X.

Inoculate the cells

Add 1.0 mL of each virus dilution into quadruplicate wells. Incubate 1 h at 37 °C, then add 100 µL EMEM–1% BSA–trypsin to each well. Incubate 4–6 days at 37 °C with 5% CO_2.

Titer determined by CPE

Discard the medium from each well into a container containing bleach. Add 0.4 mL of crystal violet–glutaraldehyde solution and leave to stain for 1 h at room temperature. Wash off the dye in running water. Allow to dry before for reading CPE.

Plating Method #2 (in-house)

Titrate virus stocks and samples:

Make tenfold dilutions of virus in sf-EMEM. Prepare dilutions in 15-mL flip-top tubes by adding 9.00 mL sf-EMEM to each tube. Use a new pipette tip for each dilution—influenza will stick to the plastic. Add 1 mL of virus to the first tube containing 9.00 mL of s-f-EMEM. Vortex the sample, then transfer 1 mL of this dilution to the next tube. Repeat for each dilution.

Prepare 24-well plates for H5N1 infection:

Remove the plates from the incubator and empty the media from the plates. Wash each well containing cells 3x with 500 µL sf-EMEM or 1XPBS. If the virus dilutions are not ready, the wells can be stored with 500 µL sf-EMEM at 37 °C under 5% CO_2 until ready.

Inoculate the cells:

After rinsing the 24-well plates with sf-EMEM or 1X PBS, add 1 mL of each virus dilution or sample into quadruplicate wells. If plates are stored in the incubator with sf-EMEM, the sf-EMEM should be discarded before inoculating the cells. Set up control wells containing

only sf-EMEM. Incubate the plates for 1 h at 37 °C with 5% CO_2. Then add 100 µL of EMEM–1% BSA–trypsin to each well. Incubate at 37 °C with 5% CO_2 for 4–6 days.

Titer determined by CPE:

On days 2–4, observe CPE under the microscope. The cells should be rounded and many should be lifted from the plate. Discard all of the media from the plates in a "BacDown" tray. Stain each of the plate's wells with 400 µL crystal violet–glutaraldehyde; stain for 1 h. Wash the dye from the plate under running water; collect the rinse for proper disposal. Allow the plates to dry before examining the CPE. The inverse of the dilution at which 50% of the wells show CPE is recorded as the tissue culture median infectious dose ($TCID_{50}$).

E-2 Reagents

- EMEM–10% FBS: 1 L EMEM , 11.5 mL Pen-Strep, 23 mL L-glutamine, 115 mL heat-inactivated fetal bovine serum.
- sf-EMEM: 1 L EMEM, 10.30 mL Pen-Strep, 21 mL L-glutamine.
- EMEM-1% BSA-trypsin: 24.24 mL serum-free EMEM, 5.0 mL 30% BSA, 750 µL of 1.0 mg/mL trypsin, 2x sterile/irradiated solution.
- Crystal Violet stain: 2.0 g crystal violet, 300 mL 50% glutaraldehyde, 2700 mL H_2O.

E-3 Virus quantification

Spearman–Karber formula

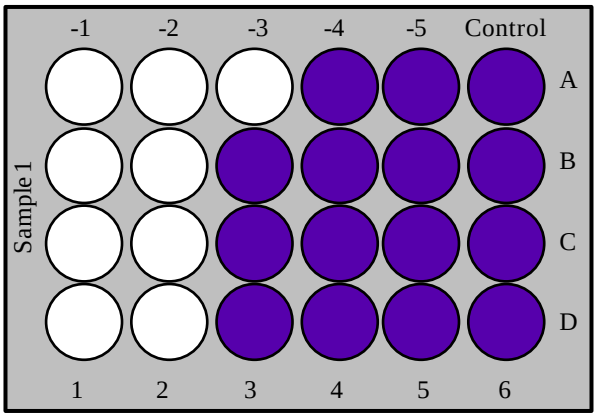
$$\boldsymbol{L = \log_{10} TCID_{50} \text{ titer} = X_0 - (d/2) + d * \sum r_i/n_i}$$

where:

X_0	=	$\log_{10}$ of the reciprocal of the lowest dilution at which all test inocula are positive.
d	=	$\log_{10}$ of the dilution factor (i.e., the difference between the log dilution intervals)
n_i	=	number of test inocula used at each individual dilution
r_i	=	number of positive test inocula (out of n_i).
$\sum(r_i/n_i)$	=	sum of the proportion of positive tests beginning at the lowest dilution showing 100% positive results.

E.4 Example Calculation for $TCID_{50}$ Assay

24-well plate, 1.0 mL of sample inoculated into each well



Purple wells—healthy cells

Clear wells—viral infected cells

L ($\log_{10}$ $TCID_{50}$) $= X_0 - (d/2) + d * \sum r_i/n_i$
$= 2 - (1/2) + 1 * \sum 4/4, 1/4$

$L = 2.75 \log_{10}$ $TCID_{50}$/mL

APPENDIX D

RT-PCR ASSAY PROTOCOL

D-1 Procedure

1. Perform the extraction with a QIAamp® MinElute® Virus Spin Kit (cat. no. 57704).
2. In the assay preparation area, label a sterile, nuclease-free, 1.5-mL tube for each reaction master mix to be prepared.
3. Calculate the amount of each reagent to be added to the tube for each master mix.
4. In a sterile, labeled, 1.5-mL tube, prepare a master mix for each marker set tested. First calculate the amount of each reagent to be added for each primer–probe set reaction master mix.
5. In the master mix preparation area, dispense reagent into labeled 1.5-mL microcentrifuge tubes. After addition of the water, mix reaction mixtures by pipetting up and down.
6. Centrifuge for 5 s to collect contents at bottom of the tube, then place the tube in a cold rack.
7. Set up the 96-well reaction plate in a 96-well cooler rack.
8. Dispense 20 µL of the master mix into each well, going across the row.
9. Prior to moving to the nucleic acid handling area, prepare the non-reactive control in the master mix preparation area.
10. Pipette 5.0 µL of nuclease-free water into the non-reactive sample wells.
11. Gently vortex nucleic acid sample tubes for approximately 5 s. Centrifuge tubes for approximately 5 s.
12. Samples should be added by row, per setup designation. Carefully pipette 5.0 µL of the first sample into all the wells labeled for that sample. Keep other sample wells covered during addition. Change tips after each addition.
13. Once all samples have been added per template setup, place the sealing foil on the 96-well plates (LightCycler® 480 Multiwell Plate 96, 50 plates with Sealing Foils, cat no 04 729 692 001).

D-2 Instrument Setup

Note: Thermal cycling conditions are as follows:

1. Reverse Transcription (RNA to *c*DNA), 30 min at 50 °C; once.
2. Initial Denaturation, 2.0 min at 95 °C; once.
3. Amplification, Step 1 denaturing, 15 s at 95 °C.
4. Amplification, Step 2 annealing, 30 s at 55.0 °C.
5. Amplification, Step 3 extension, 30 s at 72.0 °C.
6. Perform 45 repetitions of the sequence of steps 3–5.
7. Turn on the Roche LightCycler® 480. (The on/off switch is located at the back of the instrument.)

8. Launch the LightCycler® program by double clicking on the LightCycler® 480 SW 1.5 icon on the desktop (password located in the drawer below the keyboard).
9. A new window should appear. Select *New Experiment* from *Template* from the menu.
10. Scroll down the template list until you find *APHL Flu Assay 04272009*.
11. Highlight the test, then click the checkmark to open the template.
12. Select the *Subset Editor* to tell the instrument how many samples are to be tested.
13. Click the plus sign, highlight the number of wells that need to be tested, and then click *Apply* at the bottom right hand corner.
14. Locate the *Sample Editor*, click to open.
15. Choose the subset that was created for the experiment and enter the sample identifiers under the sample name for the appropriate well.
16. Before proceeding, save the run file; select the save disc located on the right. Save in appropriate run folder designation, using the following format: (initials, year and four-digit (mmdd) date - # of Run, example: tb20090928-01).
17. Once run file is saved, select *Experiment* and *Start Run*. Note: The run should take approximately 2 h to complete.

APPENDIX E

RAW DATA

Table E-1. Influenza A/H5N1 DLD (Control) Raw Count Data $TCID_{50}$ (24-well Plates)

Microwave

10/9/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	X	X	O	X	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	X	X	O
X	O	X	O	X	O
X	O	X	O	X	O

10/12/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	X
X	O	X	O	X	O
X	X	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	X
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O

10/12/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	X
X	O	X	O	X	O
X	O	X	X	X	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	X	X	O
X	O	X	O	X	X
X	O	X	O	X	O
X	O	X	O	X	O

High Heat/Humidity

10/16/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	O	O	X	X

10/16/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O

10/16/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	O	O
X	O	X	O	O	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	O	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O

UV

11/6/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	O	O
X	O	X	O	X	O
X	O	X	O	O	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	X
X	X	X	O	X	X

11/6/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
O	O	O	O	X	O
O	O	O	O	X	O
O	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O

11/6/2009 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
O	O	O	O	X	O
X	O	X	O	O	O
X	O	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	O
O	O	O	O	X	O
O	O	O	O	X	O
X	O	X	O	X	O

11/6/2009

Stock H5N1 virus titer		
-3	-4	-5
X	X	
X	X	
X	X	
X	X	X

X = positive; O = negative.

Table E-2. Influenza A/H5N1 DLD (Decon) Raw Count Data $TCID_{50}$ (24-well Plates)

Microwave						High Heat/Humidity						UV					
10/9/2009						10/16/2009						11/6/2009					
Decon 1860s						Decon 1860s						Decon 1860s					
Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Decon 1870						Decon 1870						Decon 1870					
Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
10/12/2009						10/16/2009						11/6/2009					
Decon 1860s						Decon 1860s						Decon 1860s					
Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Decon 1870						Decon 1870						Decon 1870					
Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
10/12/2009						10/16/2009						11/6/2009					
Decon 1860s						Decon 1860s						Decon 1860s					
Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
Decon 1870						Decon 1870						Decon 1870					
Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3		Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1	neat	-1
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O
O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O	O

X = positive; O = negative.

Table E-3. Influenza A/H5N1 LSAT (Control) Raw Count Data $TCID_{50}$ (24-well Plates)

Microwave

4/20/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	X	X	X
X	X	X	X	X	X
X	X	X	O	X	X
X	X	X	O	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	X
X	O	X	X	X	O
X	X	X	O	X	O
X	O	X	O	X	X

4/27/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	X	X	X
X	X	X	X	X	O
X	O	X	O	X	O
X	O	X	X	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	O	X	X
X	X	X	O	X	O
X	X	X	X	X	O
X	X	X	X	X	X

4/30/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	X	X	O
X	X	X	X	X	O
X	O	X	O	X	O
X	O	X	X	X	X

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	O
X	O	X	X	X	O
X	O	X	X	X	X
X	X	X	X	X	O

High Heat/Humidity

4/29/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	X	X	X
X	X	X	X	X	X
X	X	X	X	X	X
X	O	X	**O**	X	X

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	X
X	O	X	O	X	O
X	O	X	X	X	O
X	O	X	O	X	O

5/4/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	X	X	O
X	X	X	O	X	X
X	X	X	X	X	X
X	X	X	O	X	X

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X		X	O	X	O
X		X	O	X	O
X		X	O	X	O
X		X	X	X	O

5/10/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	X
X	X	X	O	X	O
X	O	X	O	X	O
X	O	X	X	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	X	X	O
X	O	X	O	X	O
X	O	X	X	X	O
X	X	X	O	X	X

UV

5/11/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	O
X	O	X	X	X	O
X	O	X	O	X	O
X	X	X	O	X	X

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X		X	
X		X	X	X	
X		X	X	X	
X		X		X	

5/12/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	X
X	X	X	O	X	X
X	O	X	O	X	X
X	O	X	X	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	O
X	O	X	O	X	O
X	O	X	X	X	O
X	O	X	O	X	O

5/13/2010 — Control 1860s

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	X	X	O	X	O
X	O	X	O	X	O
X	O	X	O	X	O
X	O	X	X	X	O

Control 1870

Mask 1		Mask 2		Mask 3	
-3	-4	-3	-4	-3	-4
X	O	X	X	X	O
X	O	X	X	X	O
X	X	X	O	X	X
X	X	X	O	X	X

X = positive; O = negative.

Table E-4. Influenza A/H5N1 LSAT Decon Raw Count Data $TCID_{50}$ (24-well Plates)

Microwave

4/20/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

4/27/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

4/30/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

High Heat/Humidity

4/29/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
X	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

5/4/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

5/10/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

UV

5/11/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
X	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

5/12/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	X	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	X	O	O	O

5/13/2010 — **Decon 1860s**

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

Decon 1870

Mask 1		Mask 2		Mask 3	
neat	-1	neat	-1	neat	-1
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O
O	O	O	O	O	O

X = positive; O = negative.

LIST OF ABBREVIATIONS, ACRONYMS AND SYMBOLS

AFRL	Air Force Research Laboratory
BATM	Biological Aerosol Test Method
BSL3	biosafety level 3
CDC	Centers for Disease Control and Prevention
CI	confidence interval
CoV	coefficient of variation (standard deviation/mean)
CPE	cytopathic effect
Ct	cycle threshold
cfm	cubic feet per minute
FFR	filtering facepiece respirator
h	hours
HA	hemagglutinin (an influenza viral protein)
H1N1	influenza A virus subtype (one strain is known as “swine flu”)
H5N1	influenza A virus subtype (one strain is known as “bird flu”)
HEPA	High Efficiency Particulate Air
in	inches
Lpm	liters per minute
LSAT	Laboratory-Scale Aerosol Test System
MDCK	Madin–Darby canine kidney (cells)
MH	moist heat (disinfection)
min	minutes
mL	milliliters
NIOSH	National Institute for Occupational Safety and Health
NPT	National Pipe Thread
RT-PCR	real-time polymerase chain reaction
psi	pounds per square inch
rpm	rotations per minute
s	seconds
sf-EMEM	serum-free EMEM
sf-EMEM-p/s-g	serum-free EMEM plus penicillin, streptomycin and L-glutamine
$TCID_{50}$	tissue culture median infectious dose
TEM	transmission electron micrograph
UNMC	University of Nebraska Medical Center
UVGI	ultraviolet germicidal irradiation (253.7-nm wavelength)
μ/S	microwave/steam (disinfection)
μm	micrometer